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Towards Growth of Arbuscular Mycorrhizal Fungi Independent of a Plant Host

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When surface-sterilized spores of the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* Sy167 were germinated on agar plates in the slightly modified minimum mineral medium described by G. Bécard and J. A. Fortin (New Phytol. 108:211–218, 1988), slime-forming bacteria, identified as *Paenibacillus validus*, frequently grew up. These bacteria were able to support growth of the fungus on the agar plates. In the presence of *P. validus*, hyphae branched profusely and formed coiled structures. These were much more densely packed than the so-called arbuscule-like structures which are formed by AMF grown in coculture with carrot roots transformed with T-DNA from *Agrobacterium rhizogenes*. The presence of *P. validus* alone also enabled *G. intraradices* to form new spores, mainly at the densely packed hyphal coils. The new spores were not as abundant as and were smaller than those formed by AMF in the monoxenic culture with carrot root tissues, but they also contained lipid droplets and a large number of nuclei. In these experiments *P. validus* could not be replaced by bacteria such as *Escherichia coli* K-12 or *Azospirillum brasilense* Sp7. Although no conditions under which the daughter spores regerminate and colonize plants have been found yet, and no factor(s) from *P. validus* which stimulates fungal growth has been identified, the present findings might be a significant step forward toward growth of AMF independent of any plant host.

The roots of more than 80% of land plants are colonized by arbuscular mycorrhizal fungi (AMF). Under diverse stress conditions, the hyphae of the fungi exploit water and minerals from soils better than the roots and effectively transfer them to the plant macrosymbiont. Thus, arbuscular mycorrhiza is likely the most important symbiosis in nature. Its study is hampered by the fact that AMF are obligate biotrophs (19, 20). However, as originally described by B. Mosse and C. M. Hepper (25, 26) and subsequently elaborated in much more detail by others, some AMF, such as *Gigaspora* spp. and *Glomus intraradices*, can fulfil their life cycles up to spore formation when grown in a dual system with root tissues from either carrots (5), tomatoes (3), or clover (13). The root factors which enable the AMF to grow in this monoxenic culture have not yet been fully elucidated. When spores are placed on agar containing medium only, they germinate and form so-called runner hyphae. After some weeks, however, this growth stops, the hyphae septate, and the cytoplasm is retracted. In the monoxenic culture with carrot roots, completion of the life cycle of AMF requires the synthesis of lipids which are deposited into the newly formed spores (4) and CO_2 fixations for anabolism (6). These reactions are apparently turned on by unidentified factors produced and excreted into the medium by the plant tissues before any contact of the hyphae with a root tissue cell.

Numerous investigations have indicated that rhizosphere bacteria have strong impacts on the growth of AMF (1, 2, 18). The so-called plant growth-promoting rhizobacteria (PGPR) have been described as facilitating colonization of the plants by AMF, improving the development of the mycosymbiont, and reducing damage caused by soil-borne plant pathogens. More recently, bacteria related to the genus *Burkholderia* were found to live endosymbiotically within spores of *Gigaspora* spp., where they possibly support fungal development by means of dinitrogen fixation or other, still unresolved factors (8, 9). Budi et al. (10) isolated a *Paenibacillus* strain from the mycorrhizosphere of *Sorghum bicolor* grown with *Glomus mosseae*. This bacterium had antagonistic effects toward soil-borne fungal pathogens and stimulated AMF colonization of plants.

Like plant seeds, AMF spores can only be surface sterilized, which sometimes, but not always, leads to bacterium-free inocula. In our own experiments, when surface-sterilized *G. intraradices* Sy167 spores were germinated on agar containing the minimum mineral (M) medium basically developed by Bécard and Fortin (5), slime-forming bacteria of uniform colony appearance often developed on the surface of the agar and reproducibly stimulated the growth of *G. intraradices* up to spore formation in the absence of any plant tissue. This communication describes the growth of this fungus in the presence of the bacterium and—for comparison—with the established system of carrot roots transformed with T-DNA from *Agrobacterium rhizogenes* (Ri T-DNA-transformed carrot roots) (5, 6).

MATERIALS AND METHODS

Origin of the organisms used. The AMF *G. intraradices* Sy167 (kindly supplied by the late H. Marschner, Stuttgart-Hohenheim, Germany) was grown either axenically, monoxenically with Ri T-DNA-transformed carrot (*Daucus carota* L.) roots, or in coculture with *Paenibacillus validus* (DSZM 3037 or our own isolates).

Monoxenic cultures. A monoxenic culture of *G. intraradices* Sy167 and Ri T-DNA-transformed carrot roots (kindly donated to us by H. Vierheilig, Kiel, Germany) was obtained on 0.8% agar plates containing the slightly modified M medium of Bécard and Fortin (5). Its composition, per liter, was as follows: 731 mg of $MgSO_4 \cdot 7H_2O$, 80 mg of KNO_3, 65 mg of KCl, 4.8 mg of KH_2PO_4, 288 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 8 mg of NaFe-EDTA, 0.75 mg of KI, 3 mg of $MnCl_2 \cdot 4H_2O$, 1.3 mg of $ZnSO_4 \cdot 7H_2O$, 1.5 mg of H_3BO_3, 0.065 mg of $CuSO_4 \cdot 5H_2O$,

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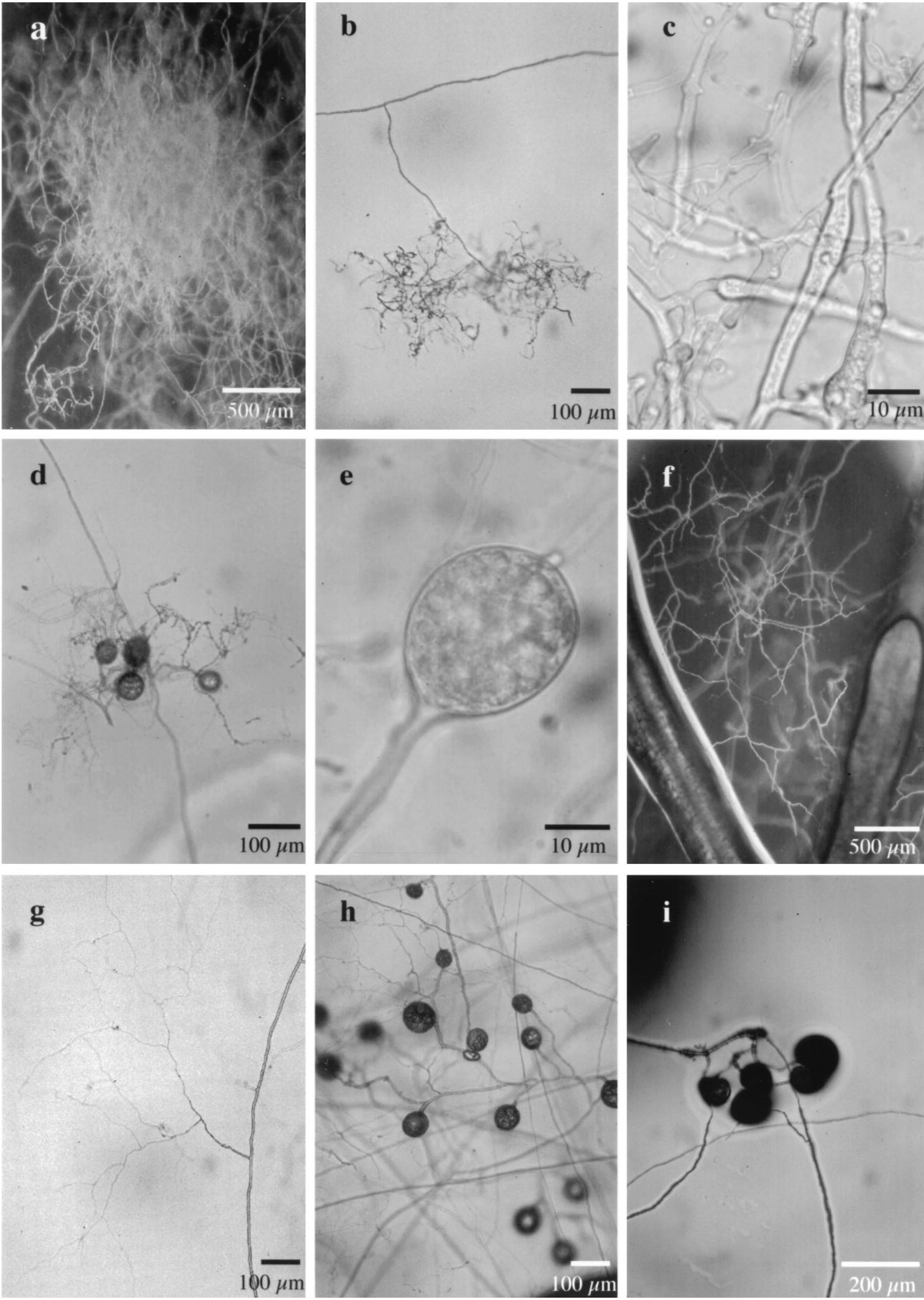
0.0012 mg of Na$_2$MoO$_4$ · 2H$_2$O, 3 mg of glycine, 0.1 mg of thiamine, 0.1 mg of pyridoxine, 0.5 mg of nicotinic acid, 50 mg of myoinositol, 1,952 mg of morpholineethanesulfonic acid (MES) buffer, 8 g of Bacto agar or 3.5 g of GelGro, and 10 g of sucrose. The pH of the medium was adjusted with KOH to 6.5 prior to sterilization at 121°C for 15 min. All plates were kept at 25°C in the dark. Petri dishes subdivided into two or three compartments (see Fig. 3) were used for enhanced spore production as described elsewhere (27).

Isolation and surface sterilization of spores. Spores of *G. intraradices* Sy167 were isolated from pot cultures with tomato (*Lycopersicon esculentum* Mill.) by wet sieving and sucrose gradient centrifugation (17). Spores were washed with sterile 0.1% MgSO$_4$ · 7H$_2$O (five times, 2 min each time) and then surface sterilized by incubation (4 h) with an antibiotic mixture (penicillin G [Sigma], streptomycin sulfate [Calbiochem], and neomycin sulfate [Sigma], each at 5 mg/liter, 2.5 mg of tetracycline hydrochloride/liter, and 50 μl of Tween 80 [Sigma]/liter in 0.1% MgSO$_4$ · 7H$_2$O). Subsequently, spores were incubated for 5 to 7 min with an NaOCl solution (from Roth; 1:50 diluted with sterile water) and further washed with 250 ml of sterile H$_2$O. Alternatively, spores were isolated from monoxenic in vitro cultures with Ri T-DNA-transformed carrot roots by solubilization of the gellan gum in M medium supplemented with 10 mM sodium citrate buffer (pH 6.0; 30°C) as described previously (15). The spores (either singly or in groups of 5 to 50) were then transferred to tricompartmented petri plates containing the slightly modified M medium in 0.8% agar in one compartment and were germinated at 25°C in the dark.

Inoculation with *P. validus*. Immediately after the first germ tubes had emerged from the spores (usually after 3 to 7 days), plates or compartments were supplemented with *P. validus* (DSMZ 3037 or DSMZ ID 99-617 or -618), which was routinely cultivated in the modified M medium at a distance of about 1.5 cm from the germinating spores.

Fungal growth under controlled atmospheric conditions. Carbon dioxide was removed from the gaseous phase of selected plates with KOH traps (6). Each KOH trap consisted of 5 ml of 1 M KOH on a small sheet of filter paper transferred into one compartment of a three-compartment petri plate which was then sealed with Parafilm. For the experiments with additional CO$_2$, nonsealed plates placed in a sealed glass container were incubated at 27°C in the dark under a continuous flow of sterile moistened atmospheric air supplemented with 2% CO$_2$.

DAPI staining of mother and daughter spores. Spores isolated from the monoxenic culture system were incubated at 27°C for 24 h with 25 mg of 4′,6′-diamidino-2-phenylindole (DAPI)/liter in liquid modified M medium. The spores were then transferred onto a glass slide and crushed with a coverslip. Daughter spores and adhering hyphae obtained in the presence of *P. validus* were excised from the surrounding agar by using a scalpel and were then treated as described above for the mother spores. The specific DAPI fluorescence was detected with a Zeiss Standard 16 microscope with fluorescence equipment, using the Zeiss optical filter system G$_{365}$FT$_{395}$LP$_{420}$.

RESULTS

***P. validus* is often a contaminant of germinating spores of *G. intraradices* Sy167.** When surface-sterilized single spores of *G. intraradices* Sy167 were germinated on 0.8% agar containing the slightly modified M medium for the growth of AMF (5), approximately 40% of the plates showed no bacterial contaminants after 4 weeks at 25°C. A mixture of nonuniform contaminants grew up on about 10% of the plates, whereas approximately 50% showed slimy, whitish to yellow-brownish, somewhat gum-like but uniform colonies. Such colonies grew

up in suspension cultures in the M medium. Sequencing of the 16S ribosomal DNA of these bacteria performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, Germany, and by us revealed their identity as *P. validus*. Two different *P. validus* isolates obtained so far by this enrichment from *G. intraradices* spores have been deposited in the DSMZ (no. ID 99-617 and ID 99-618). More remarkably, it was noted that these bacteria promoted growth of the germinated *G. intraradices* spores on the plates. Therefore, the development of these fungi in three systems—a control with spores only, a monoxenic culture with carrot root tissues, and dual culture with either of these two *P. validus* isolates or with *P. validus* DSMZ 3037—was compared (Fig. 1).

Growth of *G. intraradices* Sy167 either alone or in the presence of carrot roots. In all three systems, spores germinated within 1 week on the agar containing the M medium (5) and formed long "runner hyphae" with strong apical dominance (Fig. 1i) which occasionally branched and sometimes formed anastomoses, as described previously (11, 25). In the controls without bacteria or carrot root tissues, hyphae in the agar often curled. After 2 to 5 weeks, retraction septa became visible, growth ceased, and autolytic processes apparently occurred in the hyphae as described in detail for other fungi (3, 11, 24).

In the monoxenic culture with carrots, the root tissues were added 1 week after germination of the spores on the plates. Growth continued longer under these conditions. After 6 to 8 weeks, intimate intraradical contact between *G. intraradices* Sy167 and carrot roots had been established (Fig. 1f), as described also for other fungi (3, 13). Outside the roots, the main extension hyphae had repeatedly ramified laterally and had formed the extensively branched arbuscule-like structures (ALS) (26), also termed branched absorbing structures (BAS) (3) (Fig. 1g). Fan-like structures were not as distinct in *G. intraradices* Sy167 as in *G. mosseae* (25; our unpublished data). However, anastomoses and arbuscules, as described for another fungal isolate (11), were clearly detectable in *G. intraradices* Sy167. New spores, mainly at the ALS, became visible after 4 to 5 weeks at the earliest and were fully matured after approximately 3 months under the growth conditions employed (Fig. 1h).

Growth of *G. intraradices* Sy167 in the presence of *P. validus*. In the third system, *G. intraradices* spores on agar plates were supplemented with *P. validus* 1 week after germination. For this, a small portion of the bacteria was scratched with an inoculating loop from the agar surface of a pure *P. validus* culture and positioned on the new plate approximately 15 mm away from the germinated *G. intraradices* spores. The following developmental pattern was the same with all three *P. validus*

FIG. 1. Development of *G. intraradices* Sy167 grown either in coculture with *P. validus*, with Ri T-DNA-transformed carrot roots, or on M medium alone. (a) Massive formation of hyphae (multiple DPC) by *G. intraradices* Sy167 grown in the presence of *P. validus* for 5 months. (b) A single DPC after 1 month of growth with *P. validus*. (c) Enlarged section of a DPC showing the details of the hyphae with the wart-like structures. (d) Spore formations at DPC seen after 5 months of growth with *P. validus*. (e) Enlarged view of a single daughter spore formed at the DPC containing lipid droplets. (f) Hyphal network *of G. intraradices* Sy167 grown in coculture with carrot roots. The photo was taken after 2 months. (g) ALS formed by *G. intraradices* Sy167 grown monoxenically with carrot roots. The photo shows that the hyphae of the ALS are much less densely packed than those in the DPC (compare with panel b). (h) Three-month-old spores of *G. intraradices* Sy167 formed after growth with carrot roots. (i) Control; *G. intraradices* Sy167 grown in M medium in the absence of carrot roots and *P. validus*. Runner hyphae are formed from the germ tubes of the mother spores seen in the center of this photo.

TABLE 1. Comparison of the sizes of the newly formed spores after growth with the Ri T-DNA-transformed carrot root system or in the presence of *P. validus*

| *G. intraradices* spores | Mean spore diam[a] (μm) ± SD in system with: | |
	Carrot root tissue	*P. validus*
Original spores used for germination	73 ± 15	73 ± 15
Newly formed after 3 mo	62 ± 15	20 ± 10
Newly formed after 5 mo	No further increase	34 ± 9
Newly formed after 9 mo	No further increase	53 ± 8

[a] The diameters of at least 50 spores were measured for each value.

isolates (Fig. 1a through e). The hyphae subtending from the spores branched laterally. Both at the ends of these branches and in the middle, patches of extensively branched hyphae became visible after approximately 2 weeks at the earliest. These ramifications (networks of branches) were much more extensive than those in the dual system with transformed carrot roots. Fourth- and even fifth-order ramifications were detectable, and the short, thin branches sometimes wound even to 360°. All this resulted in densely packed coils (DPC) (Fig. 1a and b). The surfaces of these hyphae were wart-like, giving the impression that even more branches were initiated but could not develop further (Fig. 1c). Hyphae and coils were formed both on the surface and in the interior of the agar, whereas the bacteria stayed almost completely on the surface. Formation of the coils and subsequently of the spores did not require intimate contact of the bacteria with any of the fungal structures. Hyphal elongation was unlimited within the plates. The first signs of the formation of new spores were obvious after 4 to 6 weeks. Spores became visible mainly in association with the DPC (Fig. 1d) but partly also at nonbranched hyphal regions. Formation of the spores proceeded slowly. They reached 70% of the diameter of the mother spores after 9 months (Table 1). Arbuscules were not detected at any developmental stage; this was as expected, since these systems were root free.

To show whether the stimulatory effect was *P. validus* specific, single spores were supplemented with *Escherichia coli* K-12 or with *Azospirillum brasilense* Sp7 (Table 2). Incubation with these bacteria, particularly *A. brasilense* Sp7, slightly en-

TABLE 2. Formation of AMF structures after germination of single spores on modified M medium in the presence of different bacteria[a]

| Culture or condition | No. (mean ± SD) of structures | | | |
	1st-degree ramifications	Anasto-moses	Densely packed hyphal coils	Spores (after 13 wk)
Control	4 ± 3	1 ± 1	0	0
E. coli K-12	7 ± 2	1 ± 1	0	0
A. brasilense Sp7	13 ± 5	2 ± 2	0	0
P. validus	8 ± 5	1 ± 1	5 ± 4	8 ± 7
CO_2 trap	ND[b]	ND	0	0
CO_2 trap + *P. validus*	ND	ND	5 ± 4	4 ± 5

[a] Spores germinated on agar plates supplemented with M medium. After 1 week of growth of hyphae, the bacterial culture was added. Counts (*n* = 10) were done for all fungal structures after 7 weeks, with the exception of the newly formed spores (counted 13 weeks after the germination of the spores).
[b] ND, not determined.

hanced the number of ramifications, but DPC and spore formations were observed only in systems with *P. validus*. When the plates were supplemented with a trap for CO_2 (by addition of KOH to one of the two non-fungus-occupied compartments), formation of DPC and spores was not retarded in the presence of *P. validus* (Table 2). However, addition of an extra 2% CO_2 to the plates increased the number of DPC approximately twofold when they were counted 15 to 25 days after germination of the spores (data not shown).

Properties of the spores newly formed by *G. intraradices* grown in coculture with *P. validus*. Altogether, about 60% of the germinated single spores developed further to the formation of daughter spores under these experimental conditions, and the approximately 10 different experiments (with at least five replicates in each case) performed between 1997 and 2001 gave identical results. When only a single spore was incubated with *P. validus* on each plate, the number of new spores subsequently formed varied between 1 and 16, with an average of 8 (Table 2). The new spores were whitish and translucent at first, turned to yellow-brownish after about 4 months, and had the same visible appearance as the mother spores, although they were distinctly smaller. They were apparently filled with lipid droplets (Fig. 1e). More remarkably, nuclei were detectable in the new spores (Fig. 2). DAPI staining revealed the first nuclei in the developing spores after about 2 months of growth in the presence of *P. validus*. Roughly 70 nuclei were detectable in a daughter spore when it was examined 5 months after the inoculation with *Paenibacillus*, whereas the mother spores contained about 150 nuclei. More-precise figures cannot be given due to the three-dimensional structure of the spores. However, as the average number of new spores was 8 per mother spore, new nuclei must obviously have been formed in the daughter spores. Cytoplasmic streaming, in both directions, was also detectable in the hyphae of the DPC.

It must be stressed, however, that conditions under which the spores can be grown to full size have not yet been found. A culture filtrate of *P. validus* (either fresh or boiled) was inactive in supporting growth of the fungi. The numbers of spores in the experiments with one single mother spore were, admittedly, small in coculture with *P. validus* compared to those in the dual system with carrot roots. However, when 50 to 100 spores were inoculated on the agar plates, more than 1,000 spores per single plate were counted when the experiment was terminated, and the hyphae had formed extensive anastomoses, which possibly helped in the further development of the fungi. In coculture with carrot roots, on the other hand, *G. intraradices* Sy167 exhibited profuse spore formation (Fig. 3). When only hyphae, but no fungal roots, were allowed to grow into one distal compartment, thousands of spores were formed there, as described previously (12, 27). The number of these spores was not affected by the presence or absence of sucrose in the root-free compartment (Fig. 3).

Spores formed in coculture with *P. validus* were excised from the agar with a scalpel, and either one spore or two to three spores were used to supplement 2- to 3-week-old tomato plants in sterilized quartz sand under conditions described previously (17). No colonization of the tomato roots was detected under these conditions after 3 months of growth.
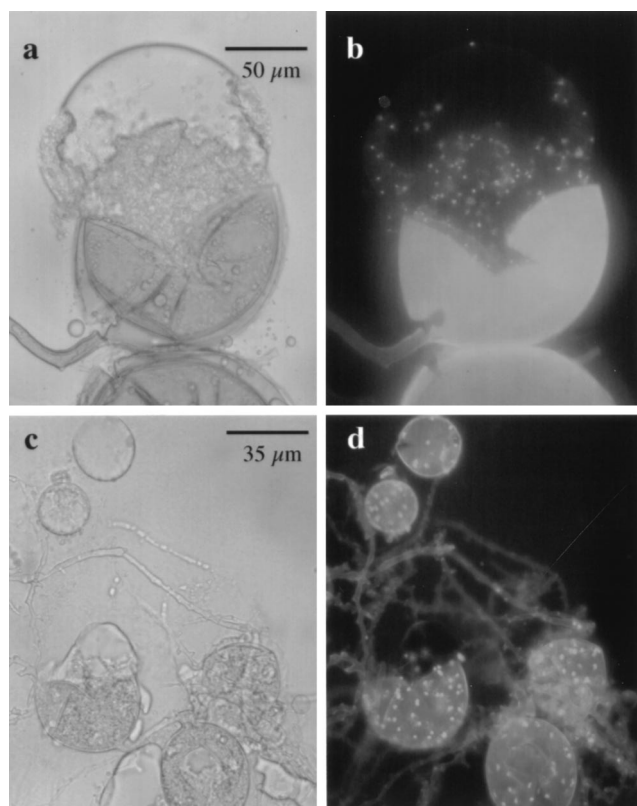
FIG. 2. Formation of nuclei in daughter spores of *G. intraradices*. (a) A mother spore of *G. intraradices* Sy167 crushed with a coverslip, observed under a light microscope. (b) The same mother spore after staining with DAPI, observed with a fluorescence microscope. (c) Daughter spores of *G. intraradices* Sy167 grown in the presence of *P. validus* DSMZ 3037, observed after being covered with a coverslip which left some spores intact and crushed others. (d) The same daughter spores after staining with DAPI, observed with a fluorescence microscope. Crushing of spores allows the nuclei to be seen more easily; otherwise, they are largely invisible under the spore wall.
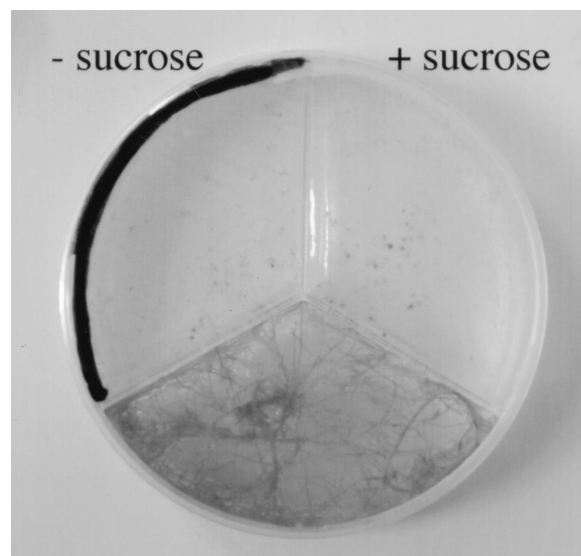

FIG. 3. Growth of *G. intraradices* Sy167 in coculture with Ri T-DNA-transformed carrot roots. In this plate, which was subdivided into three compartments, both partners were allowed to grow only in the one compartment where extensive growth is visible. Hyphae could grow into the two other compartments, which were kept free of carrot root tissue. The small spots in these two compartments show that daughter spores had formed abundantly and that this formation was independent of the presence of sucrose.

DISCUSSION

The present investigation showed that *G. intraradices* Sy167 can be grown up to spore formation, independently of any plant tissue or any plant component, but in the presence of *P. validus*. The newly formed spores contained lipid droplets and had a healthy appearance, though they were smaller than those generated in the presence of Ri T-DNA-transformed carrot roots. Spores newly formed in the presence of *P. validus* contained nuclei with DNA which was likely newly synthesized, since DNA replication has been shown to occur in the AMF *Gigaspora margarita* even in the absence of the plant host (7) and since the sum of the numbers of new nuclei formed in the daughter spores was higher than the number of nuclei in the mother spore. It should be stressed that the new spores of *G. intraradices* Sy167 did not at all resemble the first secondary spores reported by others to be formed after 1 week in the dual system of *G. intraradices* and carrot roots (11). According to these authors, the secondary spores are much smaller (20 to 30 μm in diameter) and are mainly formed terminally on short hyphal branches. Such secondary spores were not detected in the present study with the *G. intraradices* Sy167 isolate. The

spores newly formed by *G. intraradices* Sy167 in coculture with *P. validus* also did not resemble the auxiliary cells seen with *Gigaspora* or *Scutellospora* (14) or the spore-like vesicles of *Glomus clarum*, seen 1 to 2 weeks after the germination of spores of this species (13). In the present experiments, formation of daughter spores was detectable much later and preferentially, though not exclusively, at the DPC.

In symbiosis with plants, the full life cycle of AMF involves the formation of appressoria, arbuscules, and, in the case of *Glomus*, vesicles. Formation of these typical AMF structures might require intimate contact with plant roots, since they were not observed when *G. intraradices* Sy167 was grown with *P. validus*. Formation of daughter spores does not require direct contact of *P. validus* with the fungus. Thus, a chemical component (or components) might be excreted by the bacteria and utilized by the fungi. The nature of this component is not easily identified. It should be kept in mind that such factors have not been identified for the coculture of fungi and carrot roots either, despite the fact that this system was described more than 20 years ago.

In the present investigation, conditions have not yet been found under which the new spores formed in the presence of *P. validus* germinate and recolonize plants. *G. intraradices* Sy167 formed DPC when grown in the presence of *P. validus*, which could well be a result of a phytohormone excreted by the bacterium. To our knowledge, such dense hyphal coils have never been described before for any AMF grown in the absence of plant tissues. However, they seem to resemble the arbuscule-like branches formed in the external mycelium of *Glomus caledonium* grown the presence of carrot roots (see Fig. 1 in reference 23). The DPC superficially resemble the ALS (11, 26) or BAS (4) described by others, but coiling and

branching of the hyphae was much more dense, and the wart-like formations were more characteristic, in the case of the present structures. In addition, ALS are formed only after intimate intraradical contact between the fungus and the carrot roots (3). Therefore, the DPC are quite different from these ALS and also from the arbuscules formed within the roots. The term ALS has been introduced because of the gross morphological resemblance to arbuscules, which effectively take part in the exchange of metabolites between the fungal partner and the host. At present nothing is known about the metabolic activity of the newly found DPC.

This is, to our knowledge, the first report that an AMF can be grown up to spore formation independently of plant cells. We are still far from the success of the dual system with carrot roots, in which thousands of spores are formed. The present study, using the compartment system, confirms that spores of *G. intraradices* are profusely formed in the root-free compartment (12, 27), however, independently of choice of a carbon source. This compartment system appears to be selective for the generation of axenic mycorrhizal inocula and thus is not contaminated by rhizobia or other bacteria. It should be noted that this dual fungus–carrot root system does not seem to be universally applicable, since it does not support the growth of *G. mosseae* up to spore formation (16) and no root colonization was observed for *Glomus geosporum* until now (U. Hildebrandt, unpublished data).

Growth stimulation of AMF by bacteria of the *Bacillus* group, e.g., by *Bacillus mycoides* (21) or *Paenibacillus* (10), has been described previously. The stimulation of mycorrhizal colonization observed by Budi et al. (10) and the promotion of hyphal growth up to spore formation (this report) may be based on the same chemical factors produced by *Paenibacillus*. Bacteria of the *Bacillus* group do not necessarily represent new associative microorganisms or even endosymbionts within the spores (see the introduction). The *Bacillus* group comprises aerobic, spore-forming eubacteria, and this group is now subdivided into several genera (22). Bacilli occur abundantly in soils. For example, they are major constituents of the bacterial population isolated from the upper soil layer of an oak-hornbeam forest in the vicinity of Cologne (C. Rösch, Botanisches Institut, Universität zu Köln, unpublished data). Spores of such bacteria may adhere to fungal spores and may survive surface sterilization methods. Thus, it is not surprising that they grew up together with AMF in the M medium. It is, however, unexpected that they promoted growth of the fungi independent of the plant host and apparently with restricted specificity to *Paenibacillus*.

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